Antibody 9D5 recognizes oligomeric pyroglutamate amyloid- in a fraction of amyloid- deposits in Alzheimer's disease without cross-reactivity with other protein aggregates

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Abstract: Recent evidence suggests that soluble oligomeric amyloid- (A) assemblies are critically involved in the pathogenesis of Alzheimer's disease (AD). We have generated a conformation-dependent monoclonal antibody (9D5) that selectively recognizes low-molecular weight A pE3 oligomers, and demonstrated its diagnostic and therapeutic potential. Here, we further characterize the specificity of this antibody by evaluating a spectrum of neurodegeneration-related protein deposits for cross-reactivity, and by comparing the staining pattern of 9D5 with a generic A antibody that targets a linear epitope (mAb NT244), and with another conformation-dependent A antibody that selectively labels amyloid fibrils of various molecular weights (pAb OC). The 9D5 antibody does not cross-react with other aggregated protein deposits in brains of progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, Pick's disease, Parkinson's disease, dementia with Lewy bodies, multiple system atrophy, frontotemporal lobar degeneration or amyotrophic lateral sclerosis with TDP-43 inclusions, Creutzfeldt-Jakob disease, and vessel changes in Binswanger encephalopathy, demonstrating the specificity of 9D5 for A deposits. While NT244 and OC showed a comparable plaque load, 9D5 detected only approximately 15% of the total A plaque load in the entorhinal cortex, the CA1 region, and the temporal neocortex. Our study further supports a possible therapeutic advantage of 9D5 by the highly specific recognition of an epitope found only in oligomeric assemblies of A pE3 of AD patients. Moreover, selective binding to only a pathogenetically relevant fraction of A deposits serves as rationale for passive immunization with 9D5-derivatives by limiting potential side effects of vaccination due to dissolvement of existing amyloid deposits.

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Antibody 9D5 recognizes oligomeric pyroglutamate Aβ in a fraction of Aβ deposits in Alzheimer disease without cross-reactivity with other protein aggregates

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Abstract

Recent evidence suggest that soluble oligomeric Aβ assemblies are critically involved in the pathogenesis of Alzheimer disease (AD). We have generated a conformation-dependent monoclonal antibody (9D5) that selectively recognizes low-molecular weight $A\beta_{pE3}$ oligomers, and demonstrated its diagnostic and therapeutic potential. Here, we further characterize the specificity of this antibody by evaluating a spectrum of neurodegeneration-related protein deposits for cross-reactivity, and by comparing the staining pattern of 9D5 with a generic Aβ antibody that targets a linear epitope (mAb NT244), and with another conformation-dependent Aβ antibody that selectively labels amyloid fibrils of various molecular weights (pAb OC). The 9D5 antibody does not cross-react with other aggregated protein deposits in brains of progressive supranuclear palsy, corticobasal degeneration, argyrophilic grain disease, Pick`s disease, Parkinson disease, dementia with Lewy bodies, multiple system atrophy, frontotemporal lobar degeneration or amyotrophic lateral sclerosis with TDP-43 inclusions, Creutzfeldt-Jakob disease, and vessel changes in Binswanger encephalopathy, demonstrating the specificity of 9D5 for Aβ deposits. While NT244 and OC showed a comparable plaque load, 9D5 detected only approximately 15% of the total Aβ plaque load in the entorhinal cortex, the CA1 region and the temporal neocortex. Our study further supports a possible therapeutic advantage of 9D5 by the highly specific recognition of an epitope found only in oligomeric assemblies of $A\beta_{pE3}$ of AD patients. Moreover, selective binding to only a pathogenetically relevant fraction of Aβ deposits serves as rationale for passive immunisation with 9D5-derivatives by limiting potential side effects of vaccination due to dissolvement of existing amyloid deposits.

Key words: Alzheimer disease; amyloid; immunohistochemistry; conformation-dependent antibodies; oligomers; proteinopathy; pyroglutamate; neurodegenerative disease; N-truncated Abeta;

Introduction

Alzheimer disease (AD) represents a common progressive neurodegenerative disorder that is characterized neuropathologically by extracellular deposits composed of the amyloid-β (Aβ) protein and intracellular accumulation of phosphorylated tau protein [1]. Intra- or extracellular deposition of proteins is a feature of neurodegenerative diseases and serves as a molecular pathologic basis for classification as proteinopathies [2]. While immunoreactivity for Aβ or prion protein (PrP) is located predominantly extracellularly, proteins that deposit intracellularly include tau, α-synuclein, or TAR DNA Binding Protein 43 (TDP-43). Deposition of these proteins show considerable overlap [2]. Tauopathies include progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), and argyrophilic grain disease (AGD), all predominated by the 4R isoform of the tau protein, and Pick`s disease (PiD), a 3R tauopathy. Alpha-synucleinopathies comprise Parkinson`s disease (PD), dementia with Lewy bodies (DLB), and multiple system atrophy (MSA). TDP-43 proteinopathies include a group of frontotemporal lobar degenerations (FTLD-TDP), as well as sporadic amyotrophic lateral sclerosis (ALS) cases, while the most frequent form of prion disease is sporadic Creutzfeldt-Jakob disease (CJD).

Aβ peptides deposit in form of amyloid plaques or as cerebral amyloid angiopathy (CAA). Morphologically, Aβ deposits can be categorized as diffuse plaques, subpial band-like, lake-like, or fleecy deposits, and classical cored as well as so called burned-out plaques [3], reflecting a maturation process from early prefibrillar deposits to plaques with amyloid tinctorial properties. In analogy to the continuous progression and anatomical distribution pattern of neurofibrillary tangles according to Braak and Braak stages [4], Aβ pathology also follows a chronological progression course and appears in distinct susceptible brain regions, including temporal neocortex, entorhinal cortex and CA1 region [5, 6]

Several reports have demonstrated that Aβ peptides may adopt different molecular assembly states, including soluble monomers and oligomers as well as fibrillar forms [7, 8]. The latter forms represent the end product of a progressive misfolding process lastly resulting in the fomation of insoluble amyloid plaque [9, 10]. While extracellular plaques display only a minor degree of toxicity and cannot be correlated to the extent of neuronal and cognitive alterations [11], novel concepts propose the toxicity of Aβ-derived diffusible ligands (ADDLs) or soluble toxic oligomers [12, 13]. Indeed, studies in transgenic mice harboring a novel APP mutation (E693Δ), causing preferentially Aβ oligomers without any development of extracellular fibrillar Aβ deposits have shown that intracellular oligomers correlate with extensive neuronal cell death, impaired synaptic plasticity and lead to severe cognitive

decline [14]. In the past, several different oligomeric assemblies and species of Aβ have been described *in vitro* and in AD brain specimen [15, 16]. However, it is currently not clear which toxic assemblies are most relevant in AD pathogenesis [17].

Aβ displays a variety of N- and C-terminal truncations even in the same plaques [18]. N-terminal truncations modulate aggregation kinetics and resistance towards proteolytic degradation. In addition to C-terminal variations such as $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$, several N-terminal truncated Aβ versions have been identified in AD brains. Saido et al. elucidated the predominant presence of $A\beta_{pE3}$ (N-terminal truncated Aβ starting with pyroglutamate) in plaques of AD brains [19]. The formation of N-terminal pyroglutamate (pE) is catalyzed by glutamate cyclase (QC), an enzyme that was found significantly up-regulated in AD brains. Interestingly, selective overexpression of $A\beta_{pE3\text{-}42}$ within neurons induced neurodegeneration and caused a severe neurological phenotype [20]. Furthermore, pharmacologic intervention *via* QC-inhibitors resulted in reduced $A\beta_{x\text{-}40/42}$ levels, decreased plaque formation and improved memory performance [21]. Taken together, these results implicate the potential of $A\beta_{pE3\text{-}42}$ derivatives as valuable therapeutic and diagnostic target strategies in AD. In this regard, we recently generated a novel monoclonal antibody (9D5) that selectively detects oligomeric assemblies of $A\beta_{pE3}$ [22]. In contrast to other Aβ antibodies, the conformation-specific antibody 9D5 is made against a neoepitope and selectively identifies structural determinants of soluble nonfibrillar oligomers.

Insoluble deposits and diffusible oligomers from various proteinopathies may share common structural epitopes [7]. Indeed, conformation-dependent antibodies may recognize a common neo-epitope resulting in a cross-reactive response to various aggregation-prone proteins [23, 24]. Thus, in the present study, we extend our previous findings and further characterize the antibody 9D5 using an immunohistochemical approach. We 1) analyse a cohort of neurodegenerative diseases to determine potential cross-reactivity of 9D5 with other proteins; and 2) characterize and quantify 9D5 immunoreactivity of Aβ accumulation in sporadic AD and age-matched non-demented brains in comparison with a generic Aβ antibody (mAb NT244) and another conformational specific polyclonal antibody (pAb OC) [24]. The current study demonstrates the lack of cross-reactivity of 9D5 to other neurodegeneration-related protein aggregates and recognizes only a small proportion of total Aβ plaque deposition.

Materials and methods

Case selection

We performed our study on cases characterized neuropathologically according to recently published protocols [25], including seven cases with AD-related pathology in addition to two AD cases with concomitant limbic TDP-43 deposits, and three cases each of DLB, PD, MSA, PSP, PiD, CBD, AGD, FTLD-TDP, ALS, sporadic CJD (including MM-1, MV-2 and VV-2 subtypes [26];), and a single patient with Binswanger disease. Demographic data and examined anatomical regions are summarized in Table 1. Cases were obtained from the archives of the Institute of Neurology, Medical University of Vienna (Vienna, Austria) in the frame of a study ("Molecular neuropathologic investigation of neurodegenerative diseases") approved by the Ethical Committee of the Medical University of Vienna. In addition, we included ten aged-matched control (Co) patients obtained from the Netherlands Brain Bank (NBB, Amsterdam, Netherlands) and cortical frozen sections from an AD case by the University of Leipzig.

Immunohistochemistry

Formalin-fixed paraffin-embedded sections were used to detect amyloid-β (Aβ) deposits by immunhistochemistry (IHC) as described previously [27]. In brief, 4 μm paraffin sections mounted on glass slides were deparaffinized in xylene and rehydrated in ethanol. After blocking of the endogenous peroxidase activity by 0.3% hydrogen peroxide in methanol, either (A) no pretreatment; or the following epitope retrieval methods were applied: (B) 60-min in a steamer in 0.01 M citrate buffer pH 6.0; (C) 60-min in a steamer in 0.01 M citrate buffer pH 6.0 followed by 3 min formic acid (98%) treatment, and (D) 1 hour incubation in 80% formic acid. Adjacent sections of AD brain samples including temporal and entorhinal cortex, and hippocampus were immunostained using the following anti-Aβ antibodies: antibody 9D5 that detects a conformational neoepitope (mouse monoclonal, 1:100, University Medicine Goettingen and Synaptic Systems [22]), the conformation-dependent antibody OC that recognizes fibrils and soluble fibrillar Aβ oligomers of various molecular weights (rabbit polyclonal, 1:1000, generous gift of C. Glabe [24]), and the commercially available antibody NT244 detecting generic Aβ (generated by immunizing with Aβ1–16; mouse monoclonal, 1:1000, cat. no. #218211, Synaptic Systems). All primary antibodies were incubated overnight in a humidified chamber at 4°C. Sections were subsequently incubated with a horseradish peroxidase-conjugated polymer, which carries

antibodies to rabbit and mouse immunoglobulins (Envision™+/HRP, Dako, Glostrup, Denmark), and signals were visualized with the highly sensitive 3,3´-diaminobenzidine plus (DAB+) (Dako). Counterstaining was carried out with hematoxylin. As negative control, the primary antibody was replaced with blocking solution. To further evaluate the specificity of antibody 9D5, we applied the following mouse monoclonal antibodies: anti-tau AT8 (pS202, 1:200, Pierce Biotechnology, Rockford, IL, USA), anti-phospho-TDP-43 (pS409/410, 1:2,000, Cosmo Bio, Tokyo, Japan), anti-α-synuclein (1:10,000, clone 4D6, Signet, Dedham, MA, USA), and anti-PrP (1:1,000, 12F10, Cayman Chemical, Ann Arbor, MI, USA).

Frozen tissue sections from the area 7 of an AD case were prepared and stained with 9D5 (1:100) after blocking of the endogenous peroxidase activity by 0.3% hydrogen peroxide in methanol followed by 10 min formic acid (98%) treatment and developed with streptavidin/biotinyl-peroxidase method using nickel-enhanced diaminobenzidine as chromogene.

Double immunoluorescence labeling and laser confocal microscopy

Double immunolabeling was performed using the monoclonal antibody 9D5 (1:100) and the rabbit polyclonal anti-$A\beta_{40}$ antibody (1:200; Signet, Dedham, MA, USA detects the C-terminus of $A\beta_{40}$ and does not cross-react with $A\beta_{42}$ or APP) and pretreatment protocol C (see above). The fluorescence-labeled secondary antibodies were Alexa Fluor (AF) 555 donkey anti-mouse IgG (1:200; Molecular Probes, Inc., Eugene, OR, USA), and AF 488 goat anti-rabbit (1:200; Molecular Probes, Inc.). We evaluated the double fluorescence labeling with a Zeiss LSM 510 confocal laser microscope.

Morphometric assessment of Aβ deposits

Adjacent sections of 400x magnification were stained with the three Aβ antibodies (9D5, OC, and NT244) and subsequently photographed with an Olympus DP-50 camera. Extent of Aβ staining was evaluated in entorhinal cortex (EC), gyrus temporalis superior (GTS) and in the hippocampal region CA1. After maximal separation from background tissue, a fixed intensity threshold was applied to select brown-colored chromogen (DAB)-stained pixels in the same selected areas of each antibody. The images were then converted to black and white and quantitation of black pixels was performed using the Image J software (version 1.41o, National Institutes of Health). Quantitation of staining parameters were displayed as integrated optical density (arbitrary unit). In addition, we semiquantitatively evaluated different deposits (diffuse, cored plaques, primitive plaques, vascular deposits) in

subregions CA1, CA4, dentate gyrus, subiculum, entorhinal, and temporal cortices from the AD cases.

Statistical analysis

Statistical differences were calculated using one-way analysis of variance (ANOVA) followed by unpaired *t*-tests as indicated. All data are given as means ± SEM. Significance levels of unpaired *t*-tests are given as follows: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. All calculations were performed using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA, USA).

Results

Comparison of various epitope retrieval methods

The Envision™+ detection system used is a standarized and sensitive detection method [28]. In order to optimize the staining procedures, we performed comparative analysis of different antigen retrieval methods (see details in material and methods) and compared immunoreactivity of extracellular Aβ-plaques and CAA. 9D5 provided a strong and specific staining of Aβ deposits in vessels even without antigen retrieval (**Supplemental Fig. 1**). In terms of detecting extracellular Aβ deposits, the combined heat and FA antigen retrieval protocol (protocol ”C”) unequivocally demonstrated the highest staining intensity of certain Aβ plaques. For further specificity and comparative studies, we used this combined pretreatment (**Fig. 1**). In addition, we immunostained fresh-frozen tissue from a sporadic AD case: 9D5 antibody stains Aβ deposits only with formic acid pretreatment (**Supplemental Fig. 2**)

9D5 antibody presents no cross-reactivity with other aggregated protein deposits

The following pathological structures showed no immunoreactivity using 9D5 antibody (**Fig. 2**):

1) Phospho-Tau immunoreactive structures, including neurofibrillary tangles in AD and PSP, tufted astrocytes (PSP), astrocytic plaques (CBD) and oligodendroglial coiled bodies in PSP and CBD, dystrophic neurites in AD, Pick bodies in PiD, and grains in AGD.
2) α-Synuclein immunopositive Lewy bodies (brainstem and cortical) and Lewy neurites in PD and DLB, and glial cytoplasmic inclusions in MSA.

3) Phospho-TDP-43 immunoreactive neuronal cytoplasmic and neuritic deposits in FTLD-TDP, ALS, and AD with limbic TDP-43 deposits.
4) PrP immunopositive amyloid plaques, synaptic, plaque-like, and perineuronal deposits.

In addition, we did not observe any immunoreactivity associated with small vessel disease in Binswanger disease.

Antibody 9D5 immunostains only a fraction of Aβ deposits in AD brains

When evaluating different forms of Aβ deposits, we could document the maturation process of amyloid plaques with the antibody 9D5, starting from occasional intraneuronal cytoplasmic dots, seen only in the presubiculum and entorhinal cortex of the examined AD cases, through perineuronal deposition to granulo-fibrillar diffuse, primitive, and cored plaques (**Fig 3A, C, E**). However, the extent of visualized Aβ fibrils was much less in comparison with NT244 immunolabeling (**Fig. 3B, D, F**). This was further supported by double labeling with $A\beta_{40}$ antibody (**Fig. 3G**). Thus, while antibody 9D5 immunolabeled all subtypes of Aβ deposits (**Figs. 3A, C, E and 4A**), morphometric evaluation of immunodeposits in three different anatomical regions demonstrated significantly less extent and intensity of immunoreactivity using the 9D5 antibody as compared to NT244 or OC antibodies (**Fig. 4B and C**). When evaluating regional differences in the distribution of immunolabeling of different deposits, we observed no striking differences (data not shown).

In control cases, using antibodies NT244 and OC we observed diffuse parenchymal deposits, occasional cored plaques, and vessel wall deposits in five, while in another five we did not observe any immunoreactivities. Intraneuronal immunoreactivity was not seen. All other deposits were immunolabeled as seen in AD cases: only a portion of diffuse granulofibrillar Aβ deposits were immunopositive with antibody 9D5 (see **Supplemental Fig. 3**).

Discussion

Like other amyloidogenic proteins, the Aβ protein can adopt a number of different molecular assembly states, from soluble monomers and oligomers to insoluble fibrillar structures [24]. There is increasing evidence that Aβ oligomers represent the primary neurotoxic species and are closely associated with key elements of AD pathogenesis, such as synaptic dysfunction and neuron loss. Since the first description of oligomers, several alternative forms have emerged as possible disease-causing agents [29]. However, Aβ

oligomers comprise a heterogenous group (e.g. dimers, Aβ*56 and globulomers) displaying different toxic activities and are formed under various conditions [30-32].

In our view, the current challenge of antibody-based AD therapy is the elimination or neutralisation of pathological intermediate oligomers, without depleting their monomeric or higher-ordered fibrillar forms. Antibodies targeting linear Aβ epitopes are less favorable due to their inability to distinguish between physiological (monomeric) and pathological (oligomeric) derivates. Several conformation-dependent antibodies have been developed using different strategies. In addition, many oligomeric Aβ structures (and corresponding antibodies) have been reported so far, however, there is a lack of information whether these oligomers really exist in AD brain. O'Nuallain and Wetzel described conformation-dependent antibodies (W01 and W02) that preferentially bind insoluble, mature fibrils, rather than soluble oligomers and monomers [33]. Another group generated a panel of conformational polyclonal antibodies (including A11 and OC) that selectively recognize Aβ oligomers and fibrils without detecting monomeric variants [23, 24]. These antibodies react with fibrillogenic proteins from other proteinopathies. This intriguing cross-reactivity suggests the existence of common three-dimensonal structures that are independent from the linear amino acid side chain information. In contrast, conformation-specific antibodies directed against ADDLs ($A\beta_{1-42}$ oligomers) present no cross-reactivity with other amyloidogenic proteins and selectively bind Aβ oligomers and fibrils with little monomer binding [34, 35].

We followed a different approach based on the concept that N-terminally truncated Aβ species, in particular $A\beta_{pE3-x}$, represent an important element in the initiation of pathological cascades in AD [21]. It was recently shown that the presence of $A\beta_{pE3-x}$ induces neurotoxicity *in vitro* and *in vivo* [20, 36]. Furthermore, it was demonstrated that monomers of these N-terminally truncated variants are more prone to aggregate and rapidly form intermediate oligomeric assemblies (accelerated up to 250-fold compared to unmodified Aβ), implicating $A\beta_{pE3-x}$ as a putative seeding component [37]. In a first effort, we analysed the distribution of $A\beta_{pE3-x}$ in mouse models, sporadic and familial AD cases. Therefore, we generated two monoclonal antibodies directed against the linear Aβ epitope starting with pyroglutamate (mAb 2-48 and mAb 1-57). Comparison of their immunoreactivities with a generic pan-Aβ antibody (4G8) demonstrated high abundance and equal amounts of plaques staining with 4G8 and 2-48 [38]. Given the increasing interest in $A\beta_{pE3-x}$, we integrated our previous research on N-terminally modified Aβ species into the oligomer concept and

developed the conformation-dependent monoclonal antibody 9D5 that selectively recognizes oligomeric assemblies of $A\beta_{pE3-38}$ derivatives [39] . Size-exclusion chromatography followed by dot blot analysis revealed that 9D5 presents no cross-reactivity with $A\beta_{1-42}$ variants (monomers to fibrils) and selectively detects a neoepitope generated by low-molecular weight $A\beta_{pE3-x}$ oligomers (4-10mer), but not monomers and dimers [22].

In the current study, we further investigated the 9D5-specific staining properties of amyloid deposits in sporadic AD and age-matched control subjects. In line with other studies, formic acid remarkably enhanced Aβ immunostaining compared to other antigen retrieval methods, suggesting that the conformation recognized by 9D5 is also observed in fibril-dominated Aβ deposits [40, 41]. 9D5 immunolabeled only a fraction of the total Aβ immunoreactivity that was visualized by the generic antibody NT244 (recognizing a linear epitope) and the conformation-dependent antibody OC (binding to a conformational epitope). Morphological classification of 9D5 immunopositive structures revealed that apparently the whole spectrum of Aβ deposits can be detected. Oligomer-specific immunoreactivity was also associated within large cored and diffuse plaques. Nonetheless, 9D5-immunoreactivity only partially overlapped with $A\beta_{40}$-specific labeling, implicating differences in the molecular protein composition of amyloid plaques. Small punctuate clusters within neurons and scattered labeling surrounding individual cells were also seen in AD brains. Such perineuronal immunoreactivity is similar to that seen with ADDL-generated oligomeric antibodies and has been attributed to oligomer attachment to synapses (synaptic-type deposits), occuring at an early stage in AD pathology or even preclinically [42, 43].

Since the initial description of conformational-specific antibodies, an interesting issue was that they cross-react with various other fibrillogenic, disease-associated proteins. Addressing this issue, the major finding of the current study is that we could exclude cross-reactivity with the most prevalent neurodegeneration-related amyloidogenic proteins. Furthermore, our study demonstrated that 9D5 detects only a minority of AD-related plaques in sporadic cases. To highlight its implication, it must be noted, that in human patients, the first active immunization trials using AN1792 were halted in phase 2, because of severe side effects (such as meningoencephalitis) and lack of clinical efficacy. Therefore passive immunization has become a valuable alternative [44, 45]. In this context, 9D5 represents a promising therapeutic strategy in the management of AD (see review [39]).

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Table 1. Demographic data and examined anatomical regions of the cases involved in our study. Abbreviations: AD: Alzheimer disease, B&B: Braak and Braak stage, DLB: dementia with Lewy bodies, PD: Parkinson`s disease, MSA: multiple system atrophy, PSP: progressive supranuclear palsy, CBD: corticobasal degeneration, PiD: Pick`s disease, FTLD-TDP: frontotemporal lobar degeneration, ALS: amyotrophic lateralsclerosis, CJD: Creutzfeldt-Jakob disease, M: methionine, V: valine, Ent Cx: entorhinal cortex, Temp Cx: temporal cortex, SN: substantia nigra, Cbll: cerebellum.

No.	Case/Disease	Age	Sex	Examined regions
1	AD related changes (B&B III)	78	F	Hippocampus+Ent Cx+Temp Cx
2	AD related changes (B&B III)	84	M	Hippocampus+Ent Cx+Temp Cx
3	AD related changes (B&B V)	76	F	Hippocampus+Ent Cx+Temp Cx
4	AD related changes (B&B V)	81	M	Hippocampus+Ent Cx+Temp Cx
5	AD related changes (B&B V)	76	F	Hippocampus+Ent Cx+Temp Cx
6	AD related changes (B&B VI)	75	F	Hippocampus+Ent Cx+Temp Cx
7	AD related changes (B&B VI)	88	F	Hippocampus+Ent Cx+Temp Cx
8	AD related changes (B&B VI)+TDP	85	F	Hippocampus+Ent Cx+Temp Cx
9	AD related changes (B&B VI)+TDP	86	F	Hippocampus+Ent Cx+Temp Cx
10	Control-1	78	F	Hippocampus+Ent Cx+Temp Cx
11	Control-2	82	F	Hippocampus+Ent Cx+Temp Cx
12	Control-3	88	F	Hippocampus+Ent Cx+Temp Cx
13	Control-4	90	F	Hippocampus+Ent Cx+Temp Cx
14	Control-5	70	M	Hippocampus+Ent Cx+Temp Cx
15	Control-6	70	M	Hippocampus+Ent Cx+Temp Cx
16	Control-7	73	M	Hippocampus+Ent Cx+Temp Cx
17	DLB-1	78	M	Temporal Cx
18	DLB-2	82	M	Temporal Cx
19	DLB-3	81	F	Temporal Cx
20	PD-1	81	M	Mesencephalon (SN)
21	PD-2	62	M	Mesencephalon (SN)
22	PD-3	81	M	Mesencephalon (SN)
23	MSA-1	75	F	Pons
24	MSA-2	52	M	Pons
25	MSA-3	54	M	Pons
26	PSP-1	69	M	Basal Ganglia
27	PSP-2	63	M	Basal Ganglia
28	PSP-3	68	F	Basal Ganglia
29	CBD-1	70	M	Basal Ganglia
30	CBD-2	69	M	Basal Ganglia
31	CBD-3	67	M	Basal Ganglia
32	AGD-1	83	M	Hippocampus+Ent Cx+Temp Cx
33	AGD-2	85	F	Hippocampus+Ent Cx+Temp Cx
34	AGD-3	81	M	Hippocampus+Ent Cx+Temp Cx
35	PiD-1	63	F	Hippocampus+Ent Cx+Temp Cx
36	PiD-2	59	F	Hippocampus+Ent Cx+Temp Cx
37	PiD-3	70	F	Hippocampus+Ent Cx+Temp Cx
38	FTLD-TDP-1	66	M	Hippocampus+Ent Cx+Temp Cx
39	FTLD-TDP-2	55	F	Hippocampus+Ent Cx+Temp Cx
40	FTLD-TDP-3	62	F	Hippocampus+Ent Cx+Temp Cx
41	ALS-1	56	F	Spinal cord (cervical)
42	ALS-2	63	M	Spinal cord (cervical)
43	ALS-3	62	F	Spinal cord (cervical)
44	CJD MM-1	71	F	Hippocampus+Ent Cx+Temp Cx+Cbll
45	CJD MV-2	72	F	Hippocampus+Ent Cx+Temp Cx+Cbll
46	CJD VV-2	59	F	Hippocampus+Ent Cx+Temp Cx+Cbll
47	Binswanger disease	49	F	Basal Ganglia

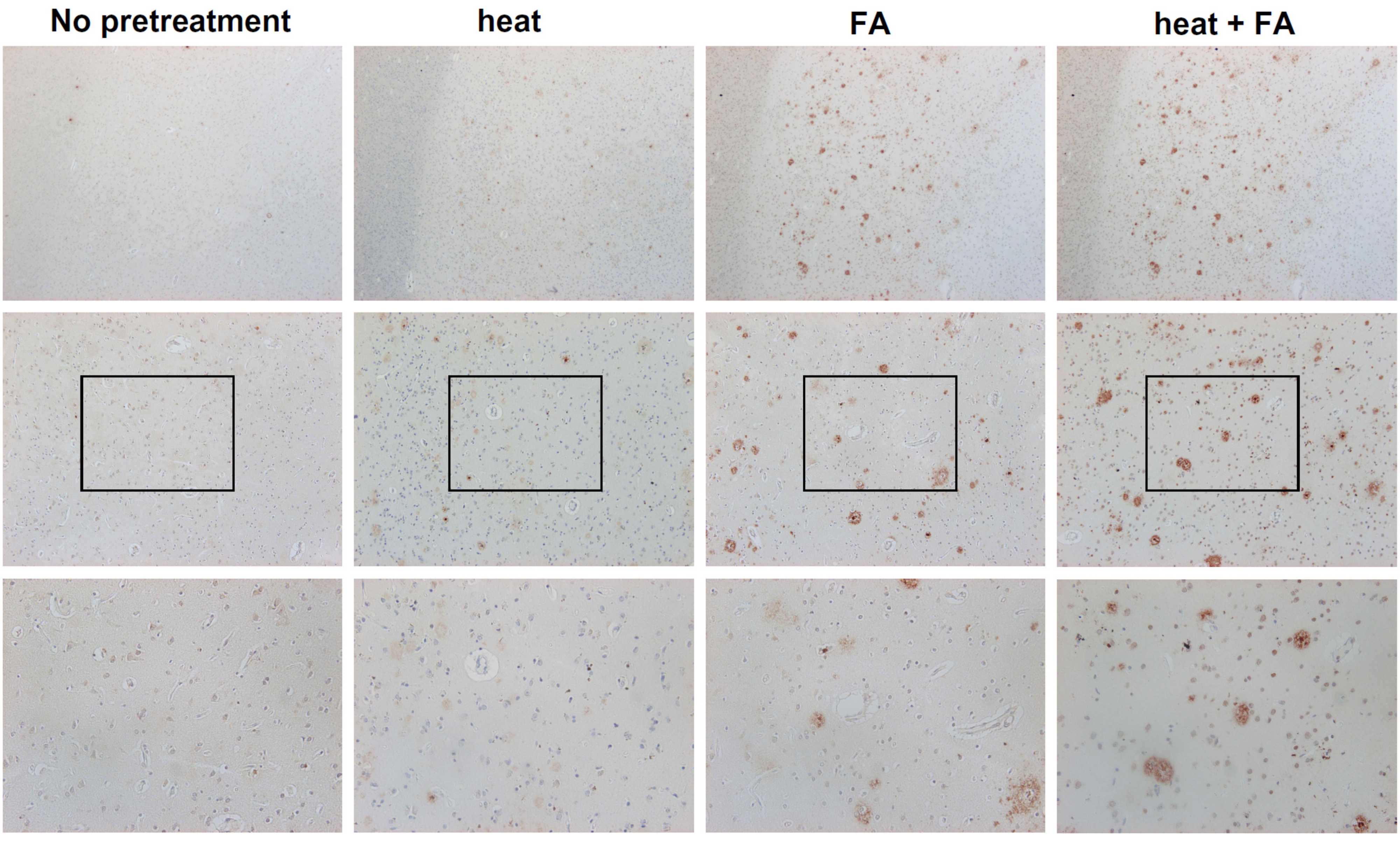
Figure legends

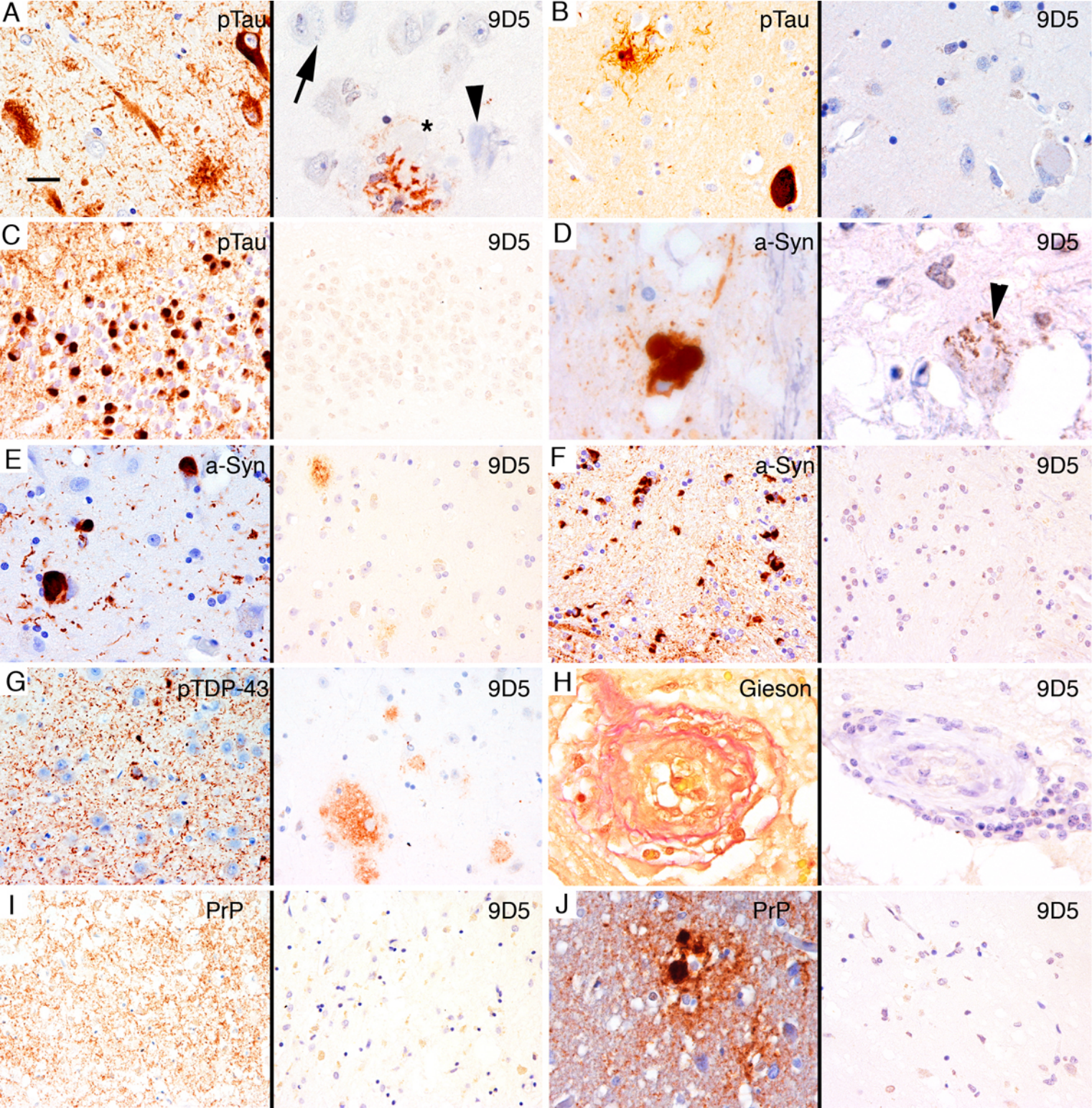
Fig. 1. Immunostaining for 9D5: comparison of different antigen retrieval methods.
Compared to no pretreatment, 60-min steam heat pretreatment in citric acid buffer pH 6 failed to improve staining of β-amyloid deposits. However, 3-min formic acid (FA) pretreatment significantly increased staining intensity of extracellular Aβ plaques. Combined treatment of heat and FA further enhanced 9D5 immunoreactivity. Scale bars: A, 500 μm; B, 200 μm; C, 100 μm.

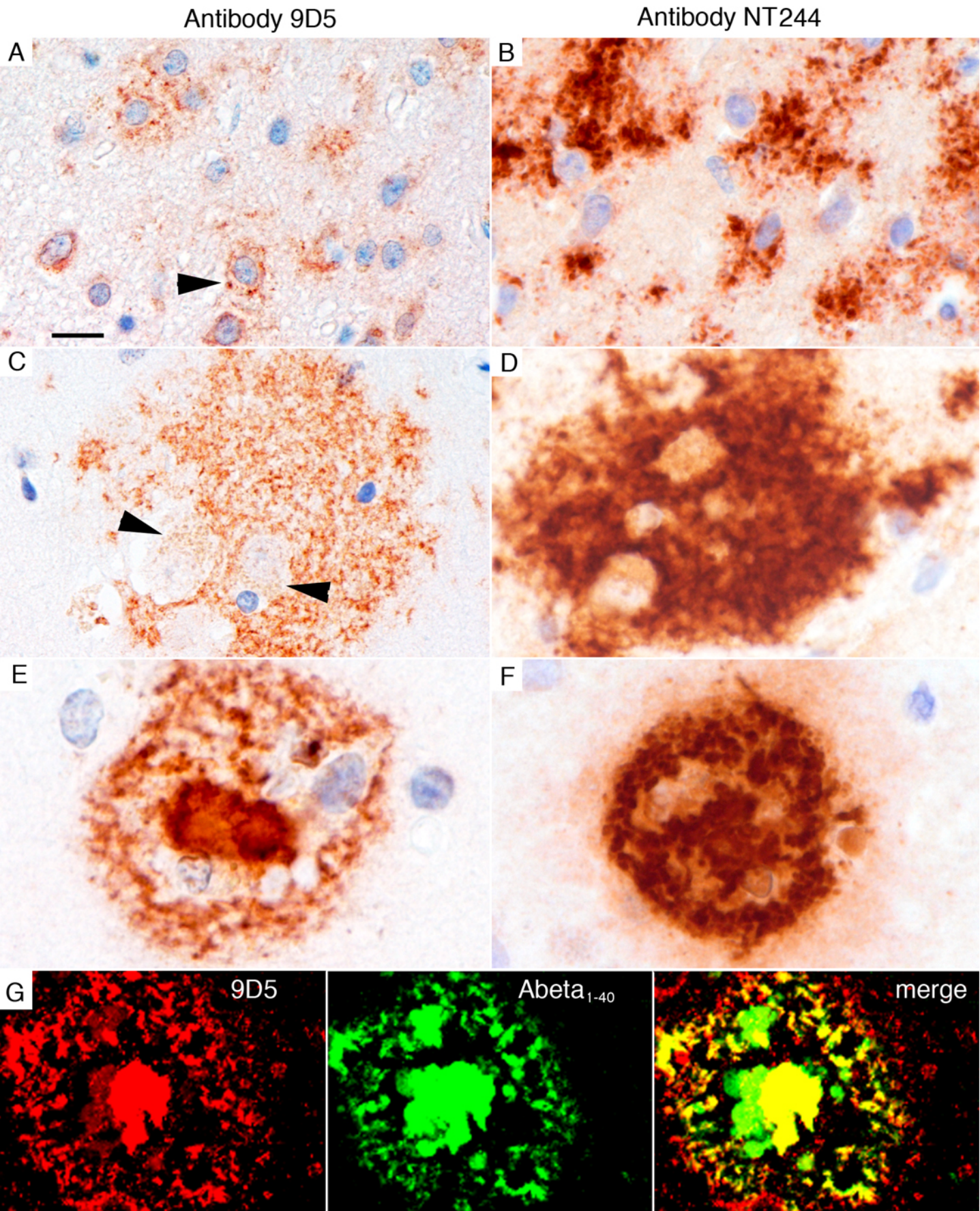
Fig. 2. Representative images of various pathological structures in neurodegenerative diseases. These lack immunopositivity for the 9D5 antibody (second and fourth column from left). Phospho-Tau immunoreactive dystrophic neurites (asterisk), granulovacuolar degeneration (arrow), neurofibrillary tangles (arrowhead) in AD (**A**) and PSP with tufted astrocytes and globular tangles (**B**); Pick bodies in Pick`s disease (**C**). α-Synuclein immunopositive brainstem (**D**; arrowhead indicates neuromelanin particles) and cortical (**E;** note the presence of Aβ deposits) Lewy bodies and Lewy neurites in PD and DLB, respectively; glial cytoplasmic inclusions in MSA (**F**). Phospho-TDP-43 immunoreactive neuronal cytoplasmic and neuritic deposits in an Alzheimer`s disease case with limbic TDP pathology (**G**; note the presence of Aβ deposits). Pathological vessels in Binswanger encephalopathy (**H**). PrP immunopositive synaptic (**I**) and plaque (**J**) deposits in sporadic Creutzfeldt-Jakob disease. Scale bar in A represents 30 μm for A-E, G and H, 50 μm for F and J, and 80 μm for I.

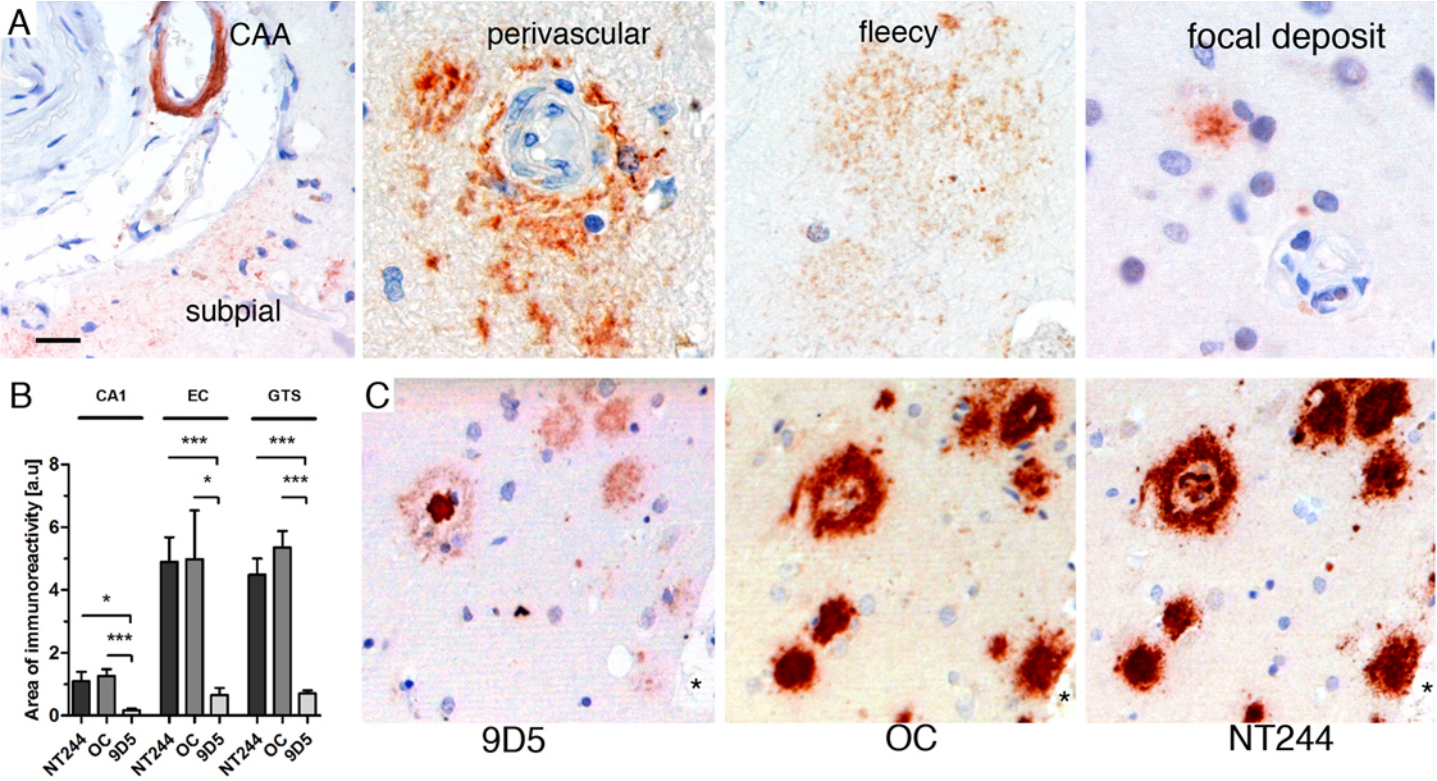
Fig. 3. Comparison of immunoreactivities using different antibodies.
Occasional intracytoplasmic dots are seen in small neurons of the entorhinal cortex using the 9D5 antibody (**A**), whereas much more perineuronal accumulation is detected by antibody NT244 (**B**). Similarly, granulofibrillar diffuse deposits (**C, D**) and the rim of cored plaques (**E, F**) are more extensively immunolabeled by NT-244 (**D, F**) than by 9D5 (**C, E**). Note the lack of intracytoplasmic staining in the neurons with prominent lipofuscin accumulation (**C**, indicated by arrowheads). Furthermore, combined immunolabeling of 9D5 and anti-$A\beta_{1-40}$ produces not fully overlapping staining patterns (**G**). Scale bar in **A** represents 15 μm for **A-D** and **G**, and 10 μm for **E**, **F**.

Fig. 4. Immunostaining for 9D5 reveals cerebral amyloid angiopathy (CAA), subpial band-like, perivascular, fleecy, and focal deposits (**A**). Quantification of morphometric data of immunolabeled deposits in the CA1 subregion of the hippocampus, in the entorhinal (EC) and temporal cortex (GTS: Gyrus temporalis superior) using antibodies 9D5, NT244, and OC (**B**). ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Note the difference in the extent of immunostained deposits as represented by adjacent sections of the temporal cortex (**C**). Scale bar in **A** represents 20 μm for the left image, 10 μm for the three images on the right, and 30 μm for **C**. Asterisks in **C** indicate the same vessel.


No pretreatment
heat
FA
heat + FA


A
pTau
9D5
B
pTau
9D5
C
pTau
9D5
D
a-Syn
9D5
E
a-Syn
9D5
F
a-Syn
9D5
G
pTDP-43
9D5
H
Gieson
9D5
I
PrP
9D5
J
PrP
9D5


Antibody 9D5
Antibody NT244
A
B
C
D
E
F
G
9D5
Abeta1-40
merge


A
CAA
subpial
perivascular
fleecy
focal deposit
B
CA1
EC
GTS
Area of immunoreactivity [a.u]
8
6
4
2
0
*
***
*
***
***
***
NT244
OC
9D5
NT244
OC
9D5
NT244
OC
9D5
C
*
*
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9D5
OC
NT244